HYPOXIC PULMONARY VASOCONSTRICTION: THE NECESSARY ROLE OF ANGIOTENSIN II

by

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B.A., San Francisco State College, 1965

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

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Deposited in the Library, San Francisco Medical Center:

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Date Librarian

Degree Conferred: 22 JUN 1974 .....

This dissertation is dedicated to Kathleen.

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ABSTRACT

THE ROLE OF ANGIOTENSIN II IN HYPOXIC PULMONARY VASOCONSTRICTION *IN VITRO* AND *IN VIVO* IN THE RAT

In isolated blood perfused rat lungs, brief periods of ventilation hypoxia (2% O_2) produce pulmonary vasoconstriction. In isolated lungs perfused with a salt-albumin solution, hypoxia produces no pulmonary vasoconstrictor responses in most lung preparations, and only minimal responses in others.

Vasoactive agents including angiotensin II, phenylephrine, epinephrine, norepinephrine, bradykinin, serotonin, and methoxamine were added to the salt-albumin perfusate to determine which substance, if any, was necessary for a pulmonary vasoconstrictor response during hypoxia.

The addition of angiotensin II to the perfusate (12 to 120 nM) resulted in marked pulmonary vasoconstriction during hypoxia in proportion to the amount of angiotensin II added (a maximal response to hypoxia occurred at 120 nM concentrations of angiotensin II). None of the other agents had the same effect, nor was their vasoactivity dependent upon angiotensin II. Angiotensin II augments the hypoxic response in doses that are themselves subpressor; tachyphylaxis to the direct pressor activity of angiotensin II is not associated with attenuation of the hypoxic response.

These results suggest that an action of angiotensin II that is separate from its pressor activity is specifically required for a significant vasoconstrictor response to hypoxia in isolated rat lungs.

The development of an effective, specific, long-lasting competitive inhibitor of angiotensin II, (1-sar, 8-ala)-angiotensin II, provided a

means of extending these observations in the isolated rat lung and in the intact animal.

In experiments with isolated rat lungs, angiotensin II-induced hypoxic pulmonary vasoconstrictor responses were prevented by prior addition of the angiotensin II inhibitor to the perfusate in concentrations ranging from 12 to 120 nM. The inhibition appears to be reversible since subsequent addition of large amounts of angiotensin II to the perfusate resulted in re-establishment of the hypoxic responses.

In experiments with intact animals, hypoxic pulmonary vasoconstriction was assessed in anesthetized rats pretreated with pentolinium tartrate to block ganglionic transmission. Pulmonary artery pressure and thoracic aorta blood flow measurements provided an estimate of pulmonary vascular resistance. The hypoxic responses seen in intact rats were reduced by systemic administration of the angiotensin II inhibitor in amounts ranging from 30 to 300 μg/kg. Angiotensin II was not measured in these experiments, but these results suggest that endogenous angiotensin II may be specifically required for a pulmonary vasoconstrictor response to hypoxia in intact animals.

INTRODUCTION

1. Literature Review

Historical

Alveolar hypoxia elicits pulmonary vasoconstriction. The first investigations relating to this effect of hypoxia were carried out in 1876 by von Lichtheim (1). While working in Carl Ludwig's laboratory in Berlin, von Lichtheim reported that anoxia caused a rise in the pulmonary artery pressure of the dog. In 1904, Nolf et al. (2) showed that the rise in pulmonary artery pressure caused by anoxia was not associated with a rise in left atrial pressure, and theorized that the effect was caused by an increase in pulmonary vascular resistance rather than a retrograde reflection of changes in the systemic arterial pressure. Plumier (3), in a separate paper published the same year, showed that the pulmonary artery response to hypoxia was independent of the concentrations of Co_2 and N_2 of the inspired gas mixture, and could be clicited by 100% H_2. Thus, it appeared that the critical factor responsible for the rise in pulmonary artery pressure was a low $P\bar{o}_2$ of the inspired gas. Wearn (4), in 1934, observed through a microscope the emptying of pulmonary surface capillaries during anoxia caused by ventilation with 100% N_2. The vasoconstriction produced by 100% N_2 occurred in the absence of acute changes in systemic pressure or cardiac output. In 1946, von Euler et al (5) postulated that the hypoxic response (hypoxic pulmonary vasoconstriction) might represent a mechanism which redistributes blood from hypoventilated areas of lung to areas with better oxygenation. This suggestion stimulated a large number of investigations on hypoxic pulmonary vasoconstriction.

Hypoxic pulmonary vasoconstriction was first demonstrated in man the following year (1947). Werko (6) and Motley et al. (7), working independently, reported experiments with human subjects in whom pulmonary vasoconstriction was induced by the inhalation of hypoxic gas mixtures.

The first studies of the hypoxic response in isolated lungs were made by Nisell (8) in 1948. Utilizing isolated cat lungs, he showed that alveolar hypoxia produced reversible increases in pulmonary artery blood pressure. This was an important observation because it demonstrated that the increased pulmonary artery pressure was a reflection of pulmonary artery vasoconstriction caused by a mechanism intrinsic to the lung itself. The mechanism was independent of nervous activity, hormonal effects, and changes in cardiac output. Since Nisell's observation in 1948, hypoxic pulmonary vasoconstriction has been demonstrated in isolated lungs from several mammalian species including dog (9), cat (10), rabbit (11) and rat (12).

Hypoxic States

It is interesting to speculate on how the hypoxic response might play a role in normal pulmonary function and in cardiovascular disease states. Chronic bronchitis is an example of a pulmonary disease in which regions of the lung become hypoxic (13). Local vasoconstriction in these areas would tend to redistribute blood away from them and help to restore normal ventilation/perfusion ratios in the lung. Söderholm et al. (14) has shown that the hypoxemia seen in patients with ventilation-perfusion abnormalities, caused by hypoventilated areas of lung, is increased by administration of a vasodilator (acetylcholine). He postulates that the vasodilator increased the degree of shunting by dilating pulmonary vessels in the diseased areas which had been constricted by alveolar hypoxia.

The first reports of high altitude pulmonary hypertension were made by Rotta et al. (15) in 1956. Since then it has been recognized that both man and animals develop pulmonary hypertension at high altitude, and that the degree of hypertension is related to the severity of the hypoxia. The elevated pulmonary artery pressure is caused by vasoconstriction, and is lowered by the administration of oxygen (16). This is an example of an undesirable effect - pulmonary hypertension - caused by the pulmonary vasoconstrictor mechanism.

Another undesirable effect, during generalized alveolar hypoxia, is cor pulmonale. Patients with Pickwickian syndrome chronically hypoventilate, and because of this develop pulmonary hypertension and subsequent cor pulmonale (17). Levy et al. (18) has reported that upper airway obstruction in children, due to hypertrophied adenoids, can result in chronic alveolar hypoxia and cor pulmonale. Patients with extensive chronic bronchitis develop cor pulmonale that is thought to be secondary to severe alveolar hypoxia (13,19). Prolonged generalized alveolar hypoxia results in pulmonary hypertension, hypertrophy of muscular pulmonary arterioles, and right ventricular hypertrophy. These structural changes tend to partially maintain the elevated pulmonary pressure during acute administration of oxygen. However, pulmonary hypertension and the structural changes seen in chronic bronchitis and at high altitude are reversed by chronic administration of oxygen (13,16).

An example of the role of the hypoxic response in the normal regulation of pulmonary vascular resistance is in the fetal lung. The high pulmonary vascular resistance of the fetal lung appears to be due to active vasoconstriction. Vasodilation with acetylcholine, or increasing the oxygen partial pressure of fetal blood will result in a partial rever-

sal of the high resistance (20). Conversely, a decrease in the partial pressure of oxygen in fetal blood will result in an increase in the pulmonary vascular resistance (21). After birth, ventilation with a gas containing a low partial pressure of oxygen tends to maintain the high pulmonary artery pressure and the fetal pattern of blood flow through the heart and lungs. However, ventilation with a gas containing a normal partial pressure of oxygen allows the normal fall in pulmonary vascular resistance and the normal change over from fetal to newborn pattern of blood flow (22).

Although it is clinically important, and despite many investigations, the mechanism underlying hypoxic pulmonary vasoconstriction remains uncertain.

Proposed Mechanisms

Over the last thirty years, a number of hypotheses have been advanced to explain the mechanism of the hypoxic response. Among the most attractive proposals are: 1) a direct effect of oxygen lack on the pulmonary vessels (23,24); 2) the local release or accumulation of lactic acid and the associated pH change (25); 3) the local release of catecholamines (26) from intact nerve pathways (27), or from chromaffin tissue (28) in the lung and 4) the local formation and/or release of other possible endogenous vasoactive mediators such as ATP, bradykinin, serotonin, and particularly histamine (29).

Bergofsky et al. (1967) (24) have shown that pulmonary artery smooth muscle loses potassium when incubated in Ringer's solution with a low P_{O_2}. The loss of potassium was not seen in the control vessels taken from the periphery of the systemic circulation. These authors postulate that the loss of potassium causes partial depolarization of the muscle membrane sensitizing it to any form of constrictor stimulus (30). Hauge (31) has

tested this hypothesis using isolated blood-perfused rat lungs. He studied the effect of stepwise reductions in the transcellular potassium gradient by adding potassium to the perfusate. Small reductions of K_{in}/K_{out} (the transcellular potassium gradient) caused potentiation of both the hypoxic response and the pressor activity of other vasoactive agents. Large reductions of K_{in}/K_{out} resulted in sustained contraction of the pulmonary vessels. However, the sustained contraction seen with large reductions of K_{in}/K_{out} did not mimic the hypoxic response, nor did it decrease the hypoxic response as one would have expected if the response was related to a transcellular leak of potassium. Furthermore, the ability of the lung to respond to hypoxia could be separated temporally from the ability of the lung to respond to large additions of potassium. From these experiments Hauge concluded that a transcellular leakage of potassium during hypoxia could not wholly account for the hypoxic response.

Smith et al. in 1951 (32), and Lloyd in 1967 (33) showed that vascular smooth muscle strips taken from lung (vessels as small as third order pulmonary arteries) relax, as do vessels from systemic peripheral vascular beds, when the oxygen tension is lowered in the organ bath. Lloyd reported in 1968 (34) that lobar artery strips from rabbit lungs will constrict in an organ bath when subjected to a low P_{O_2}, if a layer of lung perivascular tissue is allowed to adhere to the vessels. He postulated that whereas the direct effect of hypoxia on pulmonary vessels is dilatation (as in the systemic periphery), the presence of perivascular lung tissue allowed the vessels to constrict, possibly as a consequence of the release of a chemical mediator.

Hauge (35) has suggested that if the hypoxic response is due primarily to a direct effect of low oxygen tension on the pulmonary vascular

smooth muscle, one would expect arterial hypoxemia to be as effective as alveolar hypoxia in causing the hypoxic response. He tested this hypothesis in isolated blood-perfused rat lungs by varying the arterial Po_2 and the Po_2 of the inspired gas both independently and simultaneously. He found that the effect of each stimulus depended on the relative degrees of ventilation and perfusion, but that the most potent stimulus was alveolar hypoxia. He concluded that changes in the arterial Po_2 affected the oxygen tension gradient from alveolus to arterial lumen of small resistance vessels, and thus changed the Po_2 of the alveolar gas.

The experiments cited above suggest that the hypoxic response is probably not a result of the direct effect of low oxygen tension on the pulmonary vessels, although hypoxia may directly potentiate the actual stimulus (30,31). It seems likely that the accumulation, or release from perivascular tissue, of a vasoconstrictor mediator during hypoxia may effect the hypoxic response.

Liljestrand (25), in 1958, reported that infusion of lactate into the pulmonary artery of isolated lungs resulted in a drop in perfusate pH and a concomitant rise in pulmonary artery pressure. He also found that there was a positive correlation between low perfusate pH and large hypoxic responses. In these experiments, infusion of iodoacetate prevented the increase in lactate, and also reduced the response to hypoxia. From these data he suggested that the pressor effects of hypoxia in isolated lungs is dependent upon the release or accumulation of lactic acid. However, iodoacetate inhibits many enzymes including some of those concerned with tissue respiration and its specific effect upon the hypoxic response cannot be adequately assessed (36). Duke et al. (37) have found that hypoxia produces variable changes in both blood lactate and pH, and that

although there was a positive correlation between the increase in lactate and the hypoxic response in some of their experiments, in other experiments no correlation was seen. Bjurstedt et al. (1961) (38) further examined the possible role of lactic acid accumulation and pH change in the hypoxic response. Their results were not consistent with postulated effector role of lactic acid accumulation, but did suggest that increased hydrogen ion concentration can potentiate the hypoxic response. However, they also found in these experiments that hypoxia tended to reduce the hydrogen ion concentration in the perfusate during vasoconstriction, and they were forced to conclude that another independent factor must be involved in the response. Hauge (1968) (29) showed that varying the perfusate (blood) pH between 7.40 and 7.56 in isolated rat lungs did not inhibit or augment the hypoxic response. From this he concluded that hydrogen ions probably do not act as mediators of the hypoxic response.

It has been shown that electrical stimulation of the upper thoracic sympathetic outflow nerves in experimental animals results in pulmonary vasoconstriction (39). Aviado et al. (27), in 1957, observed that in situ perfused lung lobes of dogs would exhibit hypoxic responses if the aortic and carotid chemoreceptor nerves, and the perivascular nerves to the lungs, were intact. Denervation of either nerve pathway resulted in a vasodilator response to hypoxia. He concluded that there are two kinds of pulmonary vascular responses to hypoxia, the one a reflex pulmonary vasoconstriction initiated by hypoxic stimulation of the carotid and aortic chemoreceptors, the other a local vasodilation. It has been pointed out, however, that the tone of the blood vessels was likely to be significantly different in the denervated lobe, as compared to the innervated one, due to intact baroreceptor and other potential nervous influences

on the blood vessels in the latter preparation (36). Moreover, Duke (1957) (40), and Cournand (1961) (41) have each shown that chronic sympathectomy does not prevent the hypoxic response. The strongest evidence against the requirement for intact nerve pathways for the hypoxic response comes from the observation that, as previously mentioned, significant reproducible hypoxic responses are obtained in isolated lung preparations from most mammalian species studied.

Although nerve pathways connecting the lung to the rest of the body are not required for hypoxic pulmonary vasoconstriction, it has been suggested that the putative transmitter norepinephrine, or other catecholamines, may be required. Barer (26) (1966) has reported that the hypoxic response in cat lungs perfused and ventilated in situ was abolished by dibenamine and by phenoxybenzamine. The response was also prevented by guanethidine and usually prevented by pretreatment with reserpine. She postulated that the local release of catecholamines was responsible for the hypoxic response. Logaras (42), in 1947, tested the effects of ergotamine, dihydroergotamine, yohimbine, and atropine on the hypoxic response; none blocked it. The ergot derivatives have been shown to block the response to catecholamines in the lung (43). His data argue against Barer's hypothesis. Hauge tested this hypothesis in isolated blood-perfused rat lungs. In a paper published in 1968 he reported that although either phentolamine mesylate or phenoxybenzamine completely abolished the constrictor effect of norepinephrine, neither drug could abolish the hypoxic response. Phentolamine did reduce the pressor response to hypoxia in three of ten experiments, but in one of the three, the pressor effects of ATP and bradykinin were also reduced, suggesting a nonspecific effect of phentolamine (29). In another paper published the same year, Hauge

reported that pretreatment of animals with reserpine, guanethidine, or alpha-methyl-tyrosine reduced catecholamine stores in heart tissue (used as an index of reduction of catecholamines in lung tissue) by 90% without any effect upon the hypoxic response (44). He concluded that catecholamines are relatively unimportant in the response to hypoxia.

In these same publications, Hauge suggested that other endogenous vasoactive agents may act as mediators of the hypoxic response. In the first paper (29) he tested the effects of drugs which block or potentiate the direct vasoactivity of ATP, bradykinin, serotonin, and histamine upon the hypoxic response. The ATP blocking agent 2,4-xylenol blocked the pressor effect of ATP, but did not affect the hypoxic response. The bradykinin potentiating agent bovine peptide-B greatly potentiated the direct constrictor effect of bradykinin without affecting the hypoxic response. The serotonin blocking agent methysergide bimaleate had no consistent effect on the hypoxic response, but always blocked the pressor activity of serotonin. Antihistamines from four different chemical classes were able to block the hypoxic response without interfering with the pressor effects of injected bradykinin, ATP, or serotonin. The effects of the antihistamines on the hypoxic response were dose dependent, and at the higher doses they were able to block the formation of edema caused by injection of histamine into the pulmonary artery. The direct vasoactive effect of histamine in the lung was difficult to determine because histamine in doses large enough to cause an increase in pulmonary vascular resistance also produced edema and a reduction in compliance. In this same paper Hauge also showed that both thioglycollate, which potentiates histamine release from isolated rat mast cells, and semicarbazide, a histaminase inhibitor, markedly potentiated the hypoxic responses.

In his second paper in 1968 (44), Hauge et al. used releasing agents

and inhibitors of synthesis to deplete lung stores of serotonin and histamine. Serotonin content of lung was reduced to 93% of normal by prior administration of reserpine to intact rats. Hypoxic responses of the isolated lungs of these rats were not reduced by the serotonin depletion. Lung histamine was reduced to 27% of normal by simultaneous administration of 48/80 (a histamine releasing agent) and NSD 1055 (an inhibitor of histamine synthesis) to intact rats. Isolated lungs from these rats still responded to alveolar hypoxia. However, the administration of 48/80 in vitro resulted in a 90% reduction of lung histamine levels, and a concomitant disappearance of the hypoxic response. In this latter group of lungs, the response to injected bradykinin or ATP was not reduced by the administration of 48/80. Hauge postulated that histamine is a necessary mediator of the hypoxic response, but that only a small part of the total stored lung histamine is critical, and this small pool is not depleted by the agents used in his in vivo depletion experiments.

Hauge et al. (45) have reported evidence which confirms these findings. In a series of experiments using intact anesthetized cats, they showed that 48/80 abolished the hypoxic response in each case without blocking the ability of the lung vessels to react to other vasoactive agents.

Aviado et al. (46) (1966) has reported that the histamine content of pulmonary venous blood in the dog is elevated by ventilation with 100% N_2.

Hauge, and others, have presented evidence which strongly suggests that histamine may play a role in the mechanism underlying the hypoxic response. However, all the evidence is indirect and a number of questions can be raised concerning these observations. If histamine is the agent directly causing vasoconstriction during alveolar hypoxia, one might

expect to see vasoconstriction on administration of histamine which mimics that seen during hypoxia. Hauge (29) reported that administration of histamine to isolated rat lungs caused vasoconstriction only when enough had been given to cause edema formation and a decrease in compliance. The development of pulmonary edema sufficient to cause a decreased compliance might also be sufficient to cause an increase in pulmonary vascular resistance. Hauge argues, however, that accessibility to receptor sites may be an important determinant for the quantitative effect, and that the effect of endogenous release of histamine on receptor sites may not be equivalent to the effect of exogenous administration of histamine on vascular receptors. With this in mind, one might expect that the administration of the histamine releasing agent 48/80, which has been shown by Hauge et al. (44) to deplete those stores of lung histamine which are postulated to be important to the hypoxic response, would cause the release of histamine from those stores and produce a vasoconstrictor response similar to that seen during hypoxia. Administration of 48/80 produced small vasoconstrictor responses (which were blocked by the prior administration of an antihistamine compound), and these did not mimic the hypoxic responses seen prior to the administration of 48/80. However, in these experiments, histamine may not have been released at a rate sufficient to cause responses similar to those seen during hypoxia.

It should be mentioned that some investigators (Nisell, 1950 (11); Duke, 1957 (40); Barer, (1963) (47)) have reported that antihistaminic agents do not reduce the hypoxic response. These investigators used smaller amounts of the agents than Hauge used, and this may explain the discrepancy. Hauge had to use large amounts of antihistamine compounds to achieve reduction of the hypoxic response; amounts which are tolerated

by isolated lungs but not by intact animals (29). He suggested that the requirement for such large amounts of antihistamines to block the hypoxic response may be due to a relative preferential accessibility of the receptors to endogenous histamine. Another possible explanation is that the antihistamines may have an effect on the hypoxic response that is separate from their antihistaminic effect.

Glazier et al. (48), using perfused dog lungs, has reported that alveolar hypoxia constricts the pre-capillary vessels, while histamine constricts the post-capillary vessels. His evidence comes from observing where in the pulmonary vascular bed the formed elements of the blood are trapped, during hypoxia and administration of histamine, in lungs perfused in both antegrade and retrograde manner. His work has been criticized because he was only able to observe the trapped elements of the blood in the surface capillaries which may not represent the rest of the lung vasculature.

The above descriptions of the hypotheses relating to hypoxic pulmonary vasoconstriction, and evidence for and against these hypotheses, are representative of the vast amount of information and conjectures that are available in the literature to date. Although the hypotheses presented here are the most popular ones, no one has shown definitive evidence that a specific agent is required in any preparation for pulmonary vasoconstriction during hypoxia. The evidence for the involvement of histamine in the hypoxic response is, despite the questions raised above, the strongest and most acceptable evidence presented for any postulated agent.

2. Introduction to Original Work

The present study was designed to provide a more direct assessment

of the role that histamine, or other vasoactive agents, may play in the pulmonary vasoconstrictor response to hypoxia. The isolated perfused rat lung preparation was chosen for this study for a number of reasons. First, isolated lung preparations satisfy all of the criteria for demonstration of primary pulmonary vasoconstrictor responses to alveolar hypoxia as outlined by Daly et al. (49). These authors suggest that five variables must be controlled in any preparation in order to demonstrate an active response of the pulmonary vasculature to any stimulus. These are: cardiac output (flow through the lung vessels); systemic back pressure changes; changes in bronchial circulation; effects of changes in ventilation on pulmonary vessels; and bronchomotor effects. Isolated lung preparations, with only the pulmonary vasculature intact, perfused at a constant volume inflow with a constant left atrial pressure, and ventilated at a constant peak inspiratory pressure with a continuous measurement of the volume of air entering the lung during inspiration (this volume changes with changes in bronchomotor tone 12), have these five variables controlled. Second, Hauge has published six papers (12,29,31,35,44,50) on the hypoxic response in isolated blood-perfused rat lungs. These extensive investigations provide a solid background of information on which to design further experimentation. Third, the rat is a laboratory animal whose isolated lungs have been shown to give marked and reproducible vascular responses to hypoxia in a preparation which is easily set up by a single investigator. This last factor allows large numbers of experiments to be performed with maximal efficiency.

Hauge has suggested that the vasoconstrictor response to hypoxia seen in isolated blood-perfused rat lungs is dependent upon an intact mechanism in the lung and an element from the blood (12). This element from

the blood may be the vasoconstrictor itself, or it may be a precursor of the actual agent. It may also be an agent which does not directly cause vasoconstriction, but instead permits another vasoactive agent to effect a response. Clearly, the blood perfusate represents another uncontrolled variable in that it contains a myriad of substances which could play a role in the mechanism of hypoxic pulmonary vasoconstriction. To try to analyze the particular role that these substances might play during hypoxia, either by direct assay of their concentration in the blood or by observing their rate of formation or change, would be a formidable task that does not guarantee direct evidence for their involvement in the hypoxic response. Another approach would be to create an artificial perfusate which contains only the essential salts, nutrients, and colloids to insure viability of the lung and vascular tissues during perfusion. This artificial perfusate could then be used as a controlled variable, and vasoactive agents or precursors could be added to it to determine their effects during hypoxia.

Most scientific investigations begin with an interesting but unexplained observation. After the initial observation is made, the researcher has a number of options open to him in how to proceed with his investigation. He may formulate a ruling theory to explain the observation, and try by all means to prove that he is right. He may decide to utilize a particular experimental model with which he feels comfortable in hopes of collecting large amounts of meaningful data that will explain his original observation. Both of these methods have built-in pitfalls in that having a ruling theory, or being method-bound, disarms the investigator and takes away his most valuable tools, an open mind and the freedom to design and carry out appropriate experiments. A third method is the most difficult

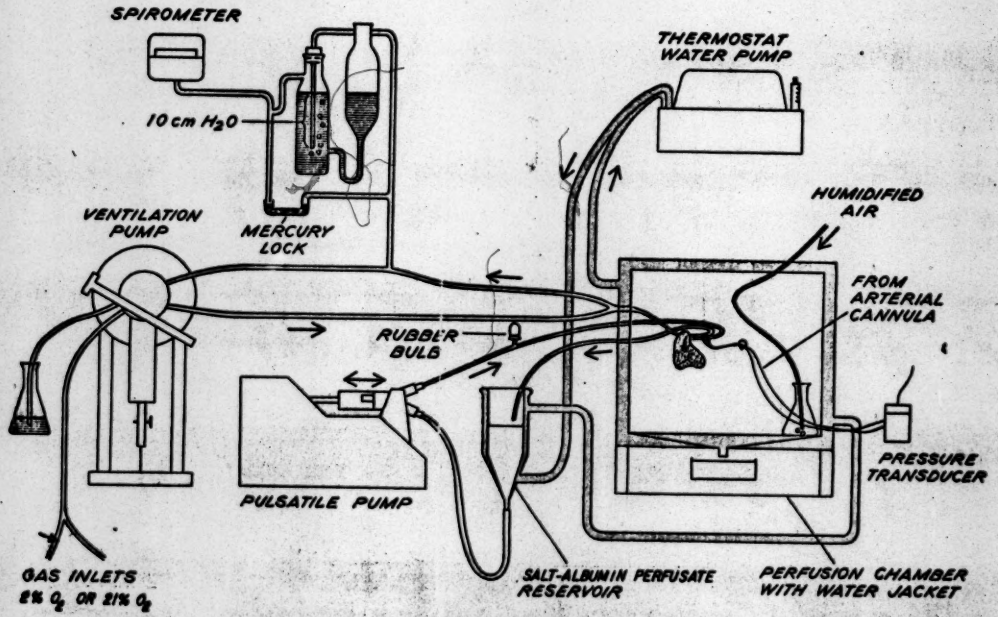
of the three, but also yields the most useful information. It is the method of multiple working hypotheses, and is designed to reduce, as much as possible, investigator bias (51,52). After the initial observation is made, the investigator formulates two or more mutually exclusive and testable hypotheses. He then designs a critical experiment, the results of which will definitely rule out one or more of the hypotheses. In this manner he constructs a logic tree with branch points that are determined by the outcome of the critical experiments. The method is not designed to prove that a particular theory is correct, but to eliminate those that are not. This is the most efficient means of obtaining meaningful information, and helps the investigator avoid becoming method-bound if the critical experiments are properly designed and carried out. This method is theoretically very good, but difficult to achieve practically. In practice, the investigator formulates hypotheses that are testable within the scope of his technical expertise, and he tries not to become emotionally attached to one of his theories.

I have tried to follow the method of multiple working hypotheses in the studies reported here. The initial observation which began these studies was that hypoxic responses were not present in an isolated lung that was perfused with an artificial perfusate. Since hypoxic responses were present in the same preparation when perfused with blood, it was obvious that an essential element was missing from the salt-albumin perfusate. The two hypotheses that I formulated to explain the observation were that an essential element was missing for general vascular reactivity, or that the missing element was specifically necessary for hypoxic pulmonary vasoconstriction. The experiments which followed differentiated between these two possibilities, and showed that angiotensin II was spe-

cifically required for the hypoxic response. The next branch point dealt with whether or not angiotensin was directly responsible for the hypoxic constriction, and the subsequent experiments suggested that it was an effect of angiotensin that is separate from its direct vasoconstrictor effect. The last series of experiments, which entailed a major change in methodology, was designed to differentiate between the questions of whether this effect of angiotensin II was peculiar to the isolated lung preparation, or whether it was an important part of the mechanism of hypoxic pulmonary vasoconstriction in vivo. The results have shown that it is part of the mechanism in vivo.

What follows is a paper, to be published in Circulation Research, that describes the experiments and methods used with the isolated rat lung perfused with an artificial perfusate. A diagram of the perfusion and ventilation arrangement used is seen in figure I.1. The results show that the addition of angiotensin II to the perfusate is specifically required for hypoxic pulmonary vasoconstriction.

Figure I.1: Isolated salt-albumin perfused rat lung preparation.



For explanation see Materials and Methods of Chapter 1.

REFERENCES

1. Lichtheim, L. von, Die Störungen des Lungenkreislaufs. August Hirschwald. Berlin 1876.

2. Nolf, P. and Plumier, L., Contribution à l'etude des réactions cardio-vasculaires de l'asphyxie chez le chien. J. Physiol. (Paris) 2. 241-253. 1904.

3. Plumier, L., La circulation pulmonaire chez la chien. Arch. Int. Physiol. 1. 176-214. 1904.

4. Wearn, J.T., A.C. Ernstene, A.W. Bromer, J.S. Barr, W.J. German and L.J. Zschiesche, The normal behavior of the pulmonary blood vessels with observations on the intermittence of the flow of blood in the arterioles and capillaries. Amer. J. Physiol. 109. 236-256. 1934.

5. Euler, U.S. von and Liljestrand, G., Observations on the pulmonary arterial blood pressure in the cat. Acta Physiol. Scand. 12. 301-320. 1946.

6. Werkö, L., The influence of positive pressure breathing on the circulation in man. Acta Med. Scand. Suppl. 193. 1947.

7. Motley, H.L., Cournand, A., Werkö L., Himmelstein A., and Dresdale, D., The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. Amer. J. Physiol. 150. 315-320. 1947.

8. Nisell, D., Effects of oxygen and carbon dioxide on the circulation of isolated and perfused lungs of the cat. Acta Physiol. Scand. 16. 121-127. 1948.

9. Daly, I. deB., Michel, C.C., Ramsay, D.J., and Waaler, B.A., Conditions governing the pulmonary vascular response to ventilation hypoxia and hypoxaemia in the dog. J. Physiol. (London) 196. 351-379. 1968.

10. Duke, H.N., Pulmonary vasomotor responses of isolated perfused cat lungs to anoxia and hypercapnia. Quart. J. Exp. Physiol. 36. 75-88. 1951.

11. Nisell, O., The action of oxygen and carbon dioxide on the bronchioles and vessels of the isolated perfused lungs. Acta Physiol. Scand. Suppl. 73, p. 21. 1950.

12. Hauge, A., Conditions governing the pressor response to ventilation hypoxia in isolated perfused rat lungs. Acta Physiol. Scand. 72. 33-44. 1968.

13. Bishop, J.M., Role of hypoxia in the pulmonary hypertension of chronic bronchitis and emphysema. Scand. J. Resp. Dis. Suppl. 77, p. 61. 1971.

14. Söderholm, B. and Widimsky, J., The effect of acetylcholine infusion on the pulmonary circulation in cases of impaired ventilation. Acta Med. Scand. 172. 219-228. 1962.

15. Rotta, A., Cánepa, A., Hurtado, A., Velásquez, T., and Chávez, R., Pulmonary circulation at sea level and at high altitudes. J. Appl. Physiol. 9. 328-336. 1956.

16. Hultgren, H.N., and Grover, R.F., Circulatory adaptation to high altitude. Ann. Rev. Med. 19. p. 119. 1968.

17. MacGregor, I.M., Block, J.A., and Ball, W.C., Serious complications and sudden death in the Pickwickian Syndrome. Johns Hopkins Med. J. 126. 279-295. 1970.

18. Levy, A.M., Tabakin, B.S., Hanson, J.S., and Narkewicz, R.M., Hypertrophied adenoids causing pulmonary hypertension and severe congestive heart failure. New Engl. J. Med. 277. 506-511. 1967.

19. Fishman, A.P., Cor pulmonale. In Harrison's Principles of Internal Medicine. 6th Ed. McGraw-Hill. p. 1316. 1970.

20. Dawes, G.S., and Mott, J.C., The vascular tone of the foetal lung, J. Physiol. (London) 164. 465-477. 1962.

21. Campbell, A.G.M., Dawes, G.S., Fishman, A.P., and Hyman, A.E., Pulmonary vasoconstriction and changes in heart rate during asphyxia in immature foetal lambs. J. Physiol. (London) 192. 93-110. 1967.

22. Cassin, S., Dawes, G.S., and Ross, B.B., Pulmonary blood flow and vascular resistance in immature foetal lambs. J. Physiol. (London) 171. 80-89. 1964.

23. Fishman, A.P., Respiratory gases in the regulation of the pulmonary circulation. Physiol. Rev. 41. 214-280. 1961.

24. Bergofsky, E.H., and Holtzman, S., A study of the mechanisms involved in the pulmonary arterial pressor response to hypoxia. Circulat. Res. 20. 506-519. 1967.

25. Liljestrand, G., Chemical control of the distribution of the pulmonary blood flow. Acta Physiol. Scand. 44. 216-240. 1958.

26. Barer, G.R., The effect of catecholamine blocking and depleting agents on the changes in the pulmonary circulation caused by hypoxia and hypercapnia. J. Physiol. (London) 186. 97-99. 1966.

27. Aviado, D.M. Jr., Ling, J.S., Schmidt, C.F., Effects of anoxia on pulmonary circulation: reflex pulmonary vasoconstriction. Amer. J. Physiol. 189. 253-262. 1957.

28. Boyd, J.D., in Adrenergic Mechanisms, Ciba. Found. Symp., (Ed.) Vane, J.R., Wolstenholme, G.E.W. and O'Connor, M., p. 63. London: Churchill.

29. Hauge, A., Role of histamine in hypoxic pulmonary hypertension in the rat: blockade or potentiation of endogenous amines, kinins, and ATP. Circulation Res. 22. 371. 1968.

30. Bergofsky, E.H., A study of the mechanism whereby hypoxia regulates pulmonary perfusion: relation between hypoxia and pulmonary artery smooth muscle potentials. Bull. Physio-Path. Resp. 2. 297-309. 1966.

31. Hauge, A., Ventilation hypoxia and pulmonary vascular resistance. Effects of changes in plasma potassium concentration. Acta Physiol. Scand. 75. 240-244. 1969.

32. Smith, D.J., and Coxe, J.W., Reactions of isolated pulmonary blood vessels to anoxia, epinephrine, acetylcholine and histamine. Amer. J. Physiol. 167. 732-737. 1951.

33. Lloyd, T.C., Influences of Po_2 and pH on resting and active tensions of pulmonary arterial strips. J. Appl. Physiol. 22. 1101-1109. 1967.

34. Lloyd, T.C., Hypoxic pulmonary vasoconstriction: role of perivascular tissue. J. Appl. Physiol. 25. 560-565. 1968.

35. Hauge, A., Hypoxia and pulmonary vascular resistance. The relative effects of pulmonary arterial and alveolar Po_2. Acta Physiol. Scand. 76. 121-130. 1969.

36. Daly, I. de B., and Hebb, C., Pulmonary vasomotor actions of ventilation hypoxia and hypercapnia. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., p. 249. 1966.

37. Duke, H., Killick, E., Marchant, J., Changes in pH of the perfusate during hypoxia in isolated perfused cat lungs. J. Physiol. 153. 413. 1960.

38. Bjurstedt, H., Liljestrand, G. and Mattell, G. In Problems of Pulmonary Circulation, Ciba Found. Study Grps. No. 8, 1961, (Ed.) de Revck, A.V.S., and O'Connor, M., p. 63. London: Churchill.

39. Daly, I. de B., and Hebb, C., Motor innervation of the lung blood vessels. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., p. 289, 1966.

40. Duke, H.N., Observations on the effects of hypoxia on the pulmonary vascular bed. J. Physiol. (London) 135. 45-51. 1957.

41. Cournand, A., In Problems of Pulmonary Circulation, Ciba Found. Study Grps. No. 8, 1961, (Ed.)de Reuck, A.V.S., and O'Connor, M., p. 42. London: Churchill.

42. Logaras, G., Further studies of the pulmonary arterial blood pressure. Acta Physiol. Scand. 14. 119-135. 1947.

43. Daly, I. de B., and Hebb, C., Catecholamines and related compounds. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., 1966, p. 202.

44. Hauge, A., and Melmon, K.L., Role of histamine in hypoxic pulmonary hypertension in the rat: depletion of histamine, serotonin, and catecholamines. Circulation Res. 22. 385-392. 1968.

45. Hauge, A., and Staub, N.C., Prevention of hypoxic vasoconstriction in cat lung by histamine releasing agent 48/80. J. Appl. Physiol. 26. 693-699. 1969.

46. Aviado, D.M., Samaněk, M., and Folle, L.E., Cardiopulmonary effects of tobacco and related substances: release of histamine during inhalation of cigarette smoke and anoxemia in the heart-lung and intact dog preparation. Arch. Environm. Health. 12. 705-711. 1966.

47. Barer, G.R., The mechanism of the increased vascular resistance caused by hypoxaemia in both collapsed and ventilated lungs. J. Physiol. (London) 169. 102. 1963.

48. Glazier, J.B., and Murray, J.F., Sites of pulmonary vasomotor reactivity in the dog during alveolar hypoxia and serotonin and histamine infusion. J. Clin. Invest. 50. 2550-2558. 1971.

49. Daly, I. de B., and Hebb, C., Criteria for the demonstration of primary pulmonary vasomotor responses. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., p. 158, 1966.

50. Haavik Nilsen, K., and Hauge, A., Effects of temperature changes on the pressor response to acute alveolar hypoxia in isolated rat lungs. Acta Physiol. Scand. 72. 111-120. 1968.

51. Platt, J.R., Strong inference. Science. 146 Number 3642. pp. 347-352. 1964.

52. Chamberlin, T.C., The method of multiple working hypothesis. Science. 148 Number 3671. pp. 754-759. 1965.

CHAPTER 1: HYPOXIA IN THE ISOLATED RAT LUNG

Inhalation of hypoxic gas mixtures elicits pulmonary vasoconstriction (1) but the direct effect of low oxygen on pulmonary artery smooth muscle is vasodilatation (2). To account for this apparent discrepancy, the release of vasomotor agents, such as histamine, from cells in the vicinity of pulmonary arterioles, or the reversible loss of potassium from vascular smooth muscle have been suggested as possible mechanisms for pulmonary vasoconstriction during hypoxia (2-5). Despite many investigations, there is no agreement on a particular mechanism.

A modification of a perfused rat lung preparation (3) was used to study the mechanism of increased pulmonary vascular resistance during alveolar hypoxia. The results indicate that angiotensin II must be present in the perfusate for hypoxia to result in pulmonary vasoconstriction.

Materials and Methods

Sprague Dawley rats of either sex (200 to 250 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg). A tracheostomy was performed and the chest opened during positive pressure ventilation. After injecting 200 U.S.P. units of heparin into the left ventricle, a stainless steel cannula was placed through the right ventricle into the pulmonary artery and a plastic cannula (PE#280) was placed through the left ventricle into the left atrium. The cannulas were secured with ligatures around the pulmonary artery and ventricles. Positive pressure ventilation and perfusion were maintained during preparation of the lungs, except for two minutes when the lungs, fully inflated, were transferred to a humidified constant temperature chamber (37°C). Pulmonary circulation was reinstituted using a Harvard Pulsatile Pump (M-1405); with a fixed

systolic-diastolic ratio of 1:2. The pump was set for a stroke rate of 120/min with a stroke volume of .17 ml.

The perfusate was an albumin and salt solution prepared by mixing 20 ml of a concentrated stock solution of electrolytes containing $CaCL_2 \cdot 2H_2O$(1.84g/1), $NaH_2PO_4 \cdot H_2O$(1.59 g/1), KCl (2.20 g/1), and NaCl (32.90 g/1), with a 20 ml of a concentrated stock solution of $NaHCO_3$ (10.46 g/1, pipetted after equilibration with 100% CO_2 for one hour), 5 ml of .1 M stock solution of D-glucose, 5 ml distilled water, and 50 ml of 10% solution (wt/vol) of salt-poor bovine albumin (Miles Pentex fraction V). The final concentration of ions in the perfusate was: Ca^{2}+5, Na^{+}140, K^{+}5.9, HCO_3^{-} 24.9, PO_4^{3-}7, Cl^{-}123.6 meq/1: glucose 5 mM, and 5% (wt/vol) albumin. The CO_2 concentration in the gas phase over the perfusate reservoir was maintained at 5% throughout the experiment to keep pH at 7.4. The perfusate was pumped through the pulmonary artery cannula from a 100 ml reservoir kept at 37°C. The first 20 ml was used to wash residual blood from the pulmonary vasculature and then discarded. The remaining 80 ml was returned to the reservoir through the left atrial cannula and recirculated. Pulmonary artery pressure was measured from a side arm on the cannula with a Statham p23AA pressure transducer connected to a Grass model 5d recorder. Under conditions of constant-mean-volume inflow, changes in pulmonary artery pressure directly reflect changes in pulmonary vascular resistance. The flow maintained in this study was 20 ml/min, which is the lower limit of normal cardiac output in the rat (6).

Positive pressure ventilation was provided by a Starling "Ideal" pump set for a 10 ml stroke volume. Valves were set so that peak inspiratory pressure did not exceed 10 cm water; expiratory pressure was 2-4 cm water. Ventilation overflow volume is that portion of the constant stroke

volume of the pump that does not enter the lung during inspiration either because compliance decreases or airway resistance increases, or both. Measurement of this volume, using a Minispirometer model 118 and the overflow arrangement described by Konzett and Rossler (7), provided a continuous assessment of changes in compliance or airway resistance resulting from edema formation, and therefore determined the suitability of the lung for continued experimentation. Lungs were rejected only after enough edema had formed to decrease compliance beyond the peak inspiratory pressure of 10 cm of water. Edema of this magnitude only developed in those lungs that were diseased at the time of utilization or that responded to too high a dose of a pressor agent by maintaining a pulmonary artery pressure above 25 mm Hg for more than a few minutes. In most cases, gentle hyperinflations given at intervals throughout the experiment were sufficient to maintain a low overflow volume.

The lungs were ventilated with a humidified gas mixture of 21% O_2, 5% CO_2, and 74% N_2, at a frequency of 60/min. To produce alveolar hypoxia the lungs were ventilated for 3 minutes with a humidified gas mixture of 2% O_2, 5% CO_2, and 93% N_2. Because albumin was added, the perfusate could not be bubbled to maintain constant pO_2, although the gas phase over the reservoir was maintained at 95% O_2.

Drugs were either injected as a bolus into the pulmonary artery cannula or directly added to the reservoir containing perfusate. All drugs were diluted in saline; the same volume of saline was administered as a control.

With the exception of methoxamine hydrochloride, all of the following chemicals were weighed (as the salt) from powdered form and diluted in saline: Phenylephrine hydrochloride (Sigma), norepinephrine hydrochloride

(Nutritional Biochemicals), bradykinin triacetate (Calbiochem), histamine dihydrochloride (B grade) (Calbiochem), serotonin creatinine sulfate (Calbiochem), epinephrine bitartrate (B grade) (Calbiochem), angiotensin II amide (CIBA), angiotensin I (synthetic A grade) (Calbiochem); methoxamine hydrochloride (aqueous) (Burroughs Wellcome).

Results

At the beginning of perfusion with the salt-albumin solution, hypoxia produced no pressor responses in most lung preparations, and only minimal pressor responses in others (increases of 2 to 3 mm Hg systolic pressure). These minimal responses, when present, could not be obtained for more than one hour after they first occurred. Subsequent experiments were designed to test whether vasoactive substances were needed in the perfusate in order to maintain vascular reactivity. It was noted that hypoxic periods produced marked pulmonary vasoconstriction after the addition of angiotensin II (Figure 1.1) and that addition of other vasoactive agents did not produce the same effect. Tables 1.1 through 1.5 tabulate data which document this effect of angiotensin II and demonstrate its specificity:

Table 1.1 (Effect of angiotensin II upon the hypoxic response) reports the results of 17 lung preparations with a total of 25 observations. In 13 of the preparations hypoxic responses were not present before the addition of angiotensin II and in the remaining 4 the average response before addition of angiotensin II was 1.1 mm Hg (mean) pressure rise. The average increase in the hypoxic response after the addition of 1 µg (12 nM) angiotensin II was 2.2 mm Hg (mean). The average increase in pulmonary artery pressure directly caused by 12 nM angiotensin II, in the

absence of tachyphylaxis, was 1.6 mm Hg (mean). Those concentrations of angiotensin II that produced only minimal or no direct pressor responses in a single lung did significantly augment the hypoxic pressor response in that lung. The dose-response relationship for angiotensin II and the hypoxic response is more clearly illustrated in Figure 1.2 (one lung preparation). Angiotensin II exerted its effect on hypoxic vasoconstriction in concentrations from 12 to 120 nM. Each addition of angiotensin II increased the subsequent hypoxic response until the maximal response was reached. Note that the hypoxic response was not present before the addition of angiotensin II to the perfusate, and although the initial addition of angiotensin II did not produce any direct vasoconstriction, hypoxia did subsequently produce a response.

Table 1.2 (Effect of angiotensin II tachyphylaxis upon the hypoxic response) reports the results of 7 lung preparations in which tachyphylaxis, to large doses (10 - 100 μg) of angiotensin II, was produced during a maximal response to hypoxia. The average direct response to 10 μg angiotensin II during tachyphylaxis was 0.7 mm Hg (mean) pressure rise. The average hypoxic response before tachyphylaxis was 6.5 mm Hg (mean) pressure rise. The average hypoxic response after tachyphylaxis was 9 mm Hg (mean) pressure rise. Thus, the pressor response to hypoxia allowed by angiotensin II was not reduced by subsequent tachyphylaxis to the direct vasoconstrictor effect of the angiotensin II.

Table 1.3 (Comparative effect of angiotensin I and angiotensin II on the hypoxic response) reports the results of 4 lung preparations with 5 sets of observations. In each set of observations angiotensin I and angiotensin II were administered (in random order) and their ability to potentiate the hypoxic response was compared. Hypoxic tests were performed

10 min after administration of either agent. The second agent was administered during the time when the hypoxic response caused by the first agent was declining. The average increase of the hypoxic response caused by 1 μg of angiotensin II was 5.2 mm Hg (mean). The average increase of the hypoxic response caused by 1 μg of angiotensin I was 1.1 mm Hg (mean). The rat lung contains an enzyme that converts approximately 20% of angiotensin I into angiotensin II during one passage through the lung, the remaining angiotensin I is converted to lower homologues of angiotensin I (8). The ability of angiotensin I to modify the hypoxic response is consistent with a partial conversion to angiotensin II, assuming the active moiety is angiotensin II and that the other degradation products of angiotensin I are not active.

Table 1.4 (Effect of other vasoactive agents upon hypoxic response) reports the effects of 7 different agents upon the hypoxic response (including catecholamines, peptides, indoleamines and imidazolamines):

a. Phenylephrine - 3 lung preparations. Average direct response to 10 μg was 1.1 mm Hg (mean) pressure rise.

b. Norepinephrine - 4 lung preparations. Average direct response to 10 μg was 1.3 mm Hg (mean) pressure rise.

c. Bradykinin - 3 lung preparations. Average direct response to 10 μg was 2 mm Hg (mean) pressure rise.

d. Histamine - 5 lung preparations. Average direct response to 10 μg was -0.3 mm Hg (mean) pressure fall.

e. Serotonin - 1 lung preparation. Average direct response to 10 μg was 0.3 mm Hg (mean) pressure rise.

f. Methoxamine - 1 lung preparation. Average direct response to 10 μg was .8 mm Hg (mean) pressure rise.

g. Epinephrine - 2 lung preparations. Average direct response to 10 μg was 2 mm Hg (mean) pressure rise.

The average change in the response to hypoxia after administration of the agents (in various amounts) was -.9 mm Hg (mean) reduction of response. The range of these changes was 0 to -5.5 mm Hg (mean) reduction of response.

Table 1.5 (Effect of angiotensin II upon response to other agents) reports the effects of angiotensin II administration upon the direct vasoactivity of 4 agents:

a. Phenylephrine - 2 lung preparations.

b. Norepinephrine - 1 lung preparation.

c. Bradykinin - 4 lung preparations.

d. Methoxamine - 1 lung preparation.

The average change in the direct vasoconstrictor response of these agents after the administration of angiotensin II, in various amounts, was -.8 mm Hg (mean) reduction in response. The range of these changes was +.5 to -2 (mean) response change.

Discussion

The data obtained from this investigation demonstrate that the hypoxic pressor response is dependent upon the presence of angiotensin II in the salt-albumin solution that is perfusing the lung (Table 1.1 and Figure 1.2). Small amounts of angiotensin II (e.g. 12 nM) allow hypoxic pressor responses for 30-60 minutes. After this time, hypoxic pressor responses return when more angiotensin II is added to the perfusate. If angiotensin II is added frequently to the perfusate the hypoxic responses persist throughout the experiment. The disappearance of the hypoxic re-

sponse, in the absence of repeated additions of angiotensin II, may be caused by inactivation of angiotensin II in the lung (9). However, the disappearance of angiotensin II from the perfusate was not directly measured in these experiments.

Three further observations concerning the effect of angiotensin II on the hypoxic pressor response resulted from this study: i) This effect is specific for angiotensin II in that agents other than angiotensin II that were tested neither produced nor potentiated the hypoxic pressor response (Table 1.4); ii) Although a wide range of doses was not tested, angiotensin II is not required for the pressor activity of other agents (Table 1.5); iii) Angiotensin II is probably not released by the hypoxic stimulus to directly cause pulmonary vasoconstriction because sub-pressor amounts of angiotensin II support the hypoxic response and tachyphylaxis to the direct pressor activity of angiotensin II does not alter the hypoxic response (Tables 1.1 and 1.2; Figure 1.2).

Thus, although angiotensin II, of all the agents tested, is specifically required for hypoxic pulmonary vasoconstriction, it probably does not directly cause it. Instead, it may permit the release or action of another agent.

Isolated lungs perfused with whole blood are responsive to hypoxia during a limited period of perfusion. The responsive period, once gone, does not return despite continuing lung viability as measured by compliance and pulmonary vascular reactivity (1). It has been suggested (1) that the disappearance of the hypoxic response in blood perfused lungs is due to changes in the lungs themselves; however, the decay of the response to hypoxia that occurs in blood perfused lungs does not occur in lungs perfused with an artificial medium containing effective concentrations of

angiotensin II. The pulmonary vasoconstrictor response to hypoxia seems to require both an intact mechanism in lung and a specific element from the blood (1). These observations, and the data in Table 1.3, suggest that a vasoconstrictor response to hypoxia may depend upon the ability of the lung to utilize directly, or form from precursors, an angiotensin II-like substance. This ability seems to be labile and has a limited effective period in isolated blood perfused lungs.

My results indicate that, of all the pressor agents tested, only angiotensin II had to be present in a salt-albumin solution perfusing an isolated rat lung in order for significant hypoxic pulmonary vasoconstriction to occur. This action of angiotensin II can be separated from its direct vasoconstrictor activity, and represents an effect of angiotensin II not previously described. The transient and minimal early response to hypoxia occasionally seen with the artificial perfusate in the absence of added angiotensin II may be an expression of this mechanism as it exists in vivo (it is therefore interesting to note that renin-like enzymes capable of forming angiotensin I from substrates have been found in tissues other than kidney, and including the rat lung) (10).

The pulmonary vasoconstriction induced by hypoxia may play a role in vivo in redistributing pulmonary blood flow from hypoxic alveoli to better oxygenated ones, if hypoxia is regional, as it is in chronic bronchitis and emphysema (11); or in the development of pulmonary hypertension or congestive heart failure if hypoxia is general, as it is in high altitude pulmonary edema or Pickwickian syndrome (12,13). The possible role of angiotensin II in these hypoxic responses has not been investigated in vivo.

Renin and renin substrate levels have been measured in blood of neo-

nates at birth and in blood of rats and people at simulated high altitude (14-16). In both instances alveolar hypoxia is associated with high pulmonary vascular resistance. At high altitude, where hypoxemia is also present, renin concentrations were found to be depressed and renin substrate levels were elevated. However, angiotensin II levels were not reported. In neonates at birth, where hypoxemia is not present, renin levels were found to be elevated and to decrease to normal levels over the subsequent three days. Again, angiotensin II levels were not reported.

Studies of the effects of changes in renin-angiotensin systems on pulmonary vascular resistance during alveolar hypoxia may lead to a better understanding of the pathogenesis of hypoxic pulmonary hypertension in vivo.

ACKNOWLEDGMENTS

I would like to thank Drs. Julius Comroe, Kenneth L. Melmon, and Henry R. Bourne for their assistance in the preparation of this manuscript.

REFERENCES

1. Hauge, A., Conditions governing the pressor response to ventilation hypoxia in isolated perfused rat lungs. Acta Physiol. Scand. 72. 33-44. 1968.

2. Lloyd, T.C., Hypoxic pulmonary vasoconstriction: role of perivascular tissue. J. Appl. Physiol. 25. 560-565. 1968.

3. Hauge, A., Role of histamine in hypoxic pulmonary hypertension in the rat. I. Blockade or potentiation of endogenous amines, kinins, and ATP. Circ. Res. 22. 371-383. 1968.

4. Hauge, A. and Melmon, K.L., Role of histamine in hypoxic pulmonary hypertension in the rat. II. Depletion of histamine, serotonin, and catecholamines. Circ. Res. 22. 385-392. 1968.

5. Bergofsky, E.H., and Holtzman, S., A study of the mechanisms involved in the pulmonary arterial pressor response to hypoxia. Circ. Res. 20. 506-519, 1967.

6. Spector, W.S., Handbook of biological data. Philadelphia, Saunders, p. 279, 1956.

7. Konzett, H., and Rössler, R., Versuchsanordnung zu untersuchungen an der bronchialmuskulatur. Arch. Exp. Pathol. Pharmakol. 195. 71. 1940.

8. Ryan, J.W., Smith, U., and Niemeyer, R.S., Angiotensin I: Metabolism by plasma membrane of lung. Science. 176. 64-66. 1972.

9. Türker, R.K., Yamamoto, M., Bumpus, F.M., and Khairallah, P.A., Lung perfusion with angiotensin I and II. Circ. Res. 28. 559-567. 1971.

10. Skeggs, L.T., Lentz, K.E., Kahn, J.R., Dorer, F.E., and Levine, M., Pseudorenin. A new angiotensin-forming enzyme. Circ. Res. 25. 451-462. 1969.

11. Bishop, J.M., Role of hypoxia in the pulmonary hypertension of chronic bronchitis and emphysema. Scand. J. Respir. Dis. (Suppl.) 77. 61-65. 1971.

12. Hultgren, H.N., and Grover, R.F., Circulatory adaptation to high altitude. Annu. Rev. Med. 19. 119-152. 1968.

13. MacGregor, I.M., Block, J.A., and Ball, W.C., Serious complications and sudden death in the Pickwickian syndrome. Johns Hopkins Med. J. 126: 279-295. 1970.

14. Hayduk, K., Krause, D.K., Huenges, R., and Unbehaun, V., Plasma renin concentration at delivery and during the newborn period in humans. Experientia. 28. 1489-1490. 1972.

15. Bing, J., and Poulsen, K., Experimentally induced changes in plasma angiotensinogen and plasma renin. Acta Pathol. Microbiol. Scand. 77. 389-398. 1969.

16. Kotchen, T.A., Hogan, R.P., Boyd, A.E., Li, T-K, Sing, H.C., and Mason, J.W., Renin, noradrenaline and adrenaline responses to simulated altitude. Clin. Sci. 44. 243-251. 1973.

Figure 1: Pulmonary pressor response to hypoxia after the addition of 12 nM angiotensin II. (taken from experiment 17, table 1)

Note: Prior to angiotensin addition, hypoxic responses were not present.

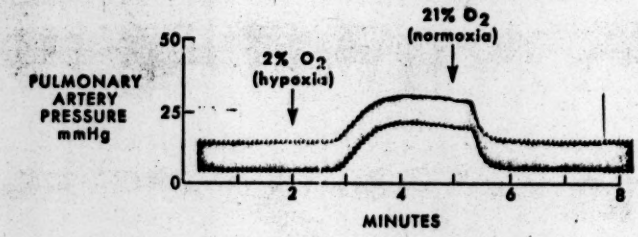


Figure42: Dose response curve for angiotensin II and the hypoxic response.

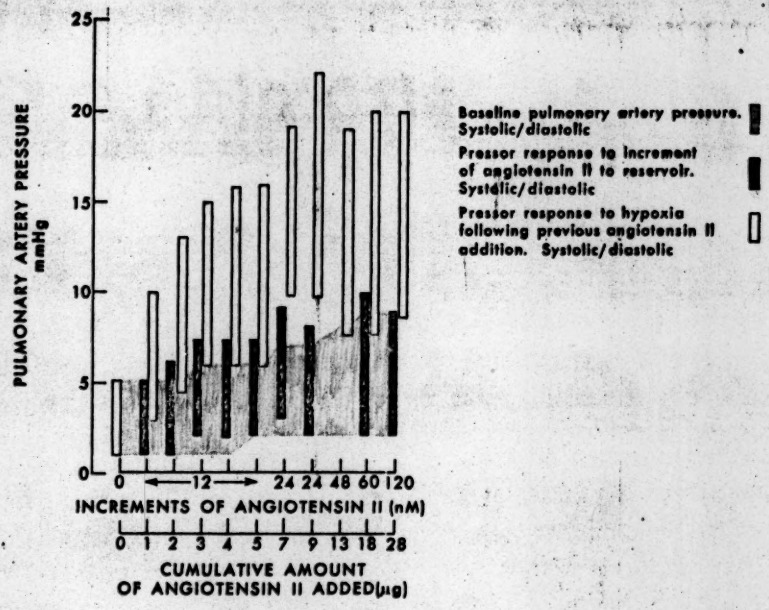


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Table 1.1 Effect of Angiotensin II upon the hypoxic response.

Experiment	Change in pulmonary artery pressure during hypoxia		Amount of Angiotensin II*		Response to Angiotensin alone‡
	Before adding Angiotensin II systolic/diastolic (mm Hg)	After adding Angiotensin II systolic/diastolic (mm Hg)	(μg)	nM	(mm Hg)
1.	0/0	10/10	10		9/7
2. a	0/0	3/1	10		13/10
b	3/1	4/2	10		8/5
3.	2/1	10/8	2		12/10
4.	0/0	4/4	1		6/3
5. a	0/0	6/5	10	120	10/7
b.	2/1	6/5	10	120	9/7
6.	0/0	4/1	10	120	5/3
7.	0/0	10/8	3	36	6/2
8.	0/0	15/11	3	36	15/8
9.	0/0	7/4	1	12	7/4
10.	3/1	10/5	1	12	2/0
11.	1/0	8/4	1	12	1/0
12.	0/0	7/4	1	12	3/1
13.	0/0	5/2	1	12	3/0
14. a	0/0	2/1	1	12	0/0
b	0/0	3/2	1	12	0/0
c	1/0	5/3	1	12	0/0
d	0/0	5/3	1	12	1/1
15. a	1/0	6/4	1	12	2/1
b	0/0	4/3	1	12	2/0
16. a	0/0	5/4	0.1	1.2	1/0
b	0/0	5/3	1	12	8/6
c	5/3	9/8	1	12	6/3 (Cont'd)

Table 1.(Cont'd)

Experiment	Change in pulmonary artery pressure during hypoxia		Amount of Angiotensin II ‡		Response to Angiotensin alone
	Before adding Angiotensin II systolic/diastolic (mm Hg)	After adding Angiotensin II# systolic/diastolic (mm Hg)	(μg)	nM	(mm Hg)
17.	0/0	16/16	1	12	17/16
18.	See Figure 2				

* The experiments with these lungs were with repeated additions of Angiotensin II (a, b, c, etc.) as shown.

‡ Added to reservoir except for experiments 1 – 4 (injected into pulmonary artery). nM concentrations based on 80 ml reservoir volume -- not calculated for pulmonary artery injections.

Hypoxic test performed after response to Angiotensin II alone had returned to baseline.

Table 2. Effect of Angiotensin II tachyphylaxis upon the hypoxic response.

Experiment	Change in pulmonary artery pressure during hypoxia		Amount of Angiotensin (μg)*	Response to Angiotensin during tachyphylaxis
	Before tachyphylaxis systolic/diastolic (mm Hg)	During tachyphylaxis systolic/diastolic (mm Hg)		
1.	10/10	10/10	20	2/2
2.	6/5	6/4	20	1/1
3.	5/2	6/2	10	4/2
4.	14/8	15/14	30	4/3
5.	9/8	12/10	20	3/2
6.	5/3	12/12	20	4/4
7.	4/2	7/6	100	0/0

* Injected into pulmonary artery

Table 3. Comparative effect of Angiotensin I and Angiotensin II on the hypoxic response.

Experiment	Increase of hypoxic pressor response above previous test		Amount* and response to					
	After adding Angiotensin I	After adding Angiotensin II	Angiotensin I			Angiotensin II		
	systolic/diastolic (mm Hg)	systolic/diastolic (mm Hg)	(µg)	(nM)	(mm Hg)	(µg)	(nM)	(mm Hg)
1.	4/2	7/6	1	10	0/0	1	12	6/3
2.	3/1	7/4	1	10	0/0	1	12	6/4
3 a.	5/3	5/3	5	50	1/1	1	12	1/1
3 b.	7/4	7/5	5	50	2/0	1	12	2/1
4.	5/3	5/3	5	50	2/1	1	12	2/1

* Added to reservoir

Table 4. Effect of other vasoactive agents upon hypoxic response.

Experiment	Change in pulmonary artery pressure during hypoxia		Amount of agent (μg) +	Response to agent alone (mm Hg)
	Before adding agent systolic/diastolic (mm Hg)	After adding agent systolic/diastolic (mm Hg)		
Phenylephrine:				
1.	0/0	0/0	20	2/0
2.	0/0	0/0	10	3/1
3.	3/2	2/1	10	2/1
Norepinephrine:				
1.	1/0	1/0	10	6/2
2.	0/0	0/0	10	7/1
3.	10/4	6/2	20	3/1
4.	12/5	10/3	50	3/1
Bradykinin:				
1.	0/0	0/0	20	4/2
2.	7/2	4/0	5	5/2
3.	7/7	6/6	10	1/0
Histamine:				
1.	0/0	0/0	15	0/0
2.	1/0	1/0	100	-3/0
3.	0/0	0/0	10	-3/0
4.	10/4	10/4	10	-2/0
5.	6/2	4/1	20	-2/0

(Cont'd)

Table 4 (Cont'd).

Experiment	Change in pulmonary artery pressure during hypoxia		Amount of agent (μg)+	Response to agent alone (mm Hg)
	Before adding agent systolic/diastolic (mm Hg)	After adding agent systolic/diastolic (mm Hg)		
Serotonin:				
1.	1/0	1/0	100	4/1
Methoxamine:				
1.	9/7	3/2	20	2/1
Epinephrine:				
1.	0/0	0/0	1	4/1
2.	0/0	0/0	20	3/1

* In these experiments, angiotensin had been given before addition of the agent being tested.

\+ Injected into pulmonary artery, with the exception of Experiment 1 — phenylephrine (20 μg added to reservoir)

Table 45. Effect of Angiotensin II upon response to other agents.

Experiment	Change in pulmonary artery pressure caused by agent		Amount of Angiotensin (μg)	Response to Angiotensin alone (mm Hg)
	Before adding Angiotensin II systolic/diastolic (mm Hg)	After adding Angiotensin II systolic/diastolic (mm Hg)		
Phenylephrine				
1.	3/1 (10 μg PA)	2/0 (10 μg PA)	3 (RES)	0/0
2.	2/1 (10 μg PA)	1/0 (10 μg PA)	2.5 (PA)	12/10
Norepinephrine:				
1.	7/1 (10 μg PA)	3/1 (10 μg PA)	3 (RES)	15/8
Bradykinin:				
1.	5/2 (5 μg PA)	5/2 (5 μg PA)	2 (RES)	4/1
2.	2/1 (10 μg PA)	2/2 (10 μg PA)	0.5 (RES)	11/10
3.	2/2 (10 μg PA)	2/1 (10 μg PA)	1 (RES)	16/12
4.	2/1 (10 μg PA)	2/1 (10 μg PA)	50.0 (PA)	0/0 (tachyphyloxis)
Methoxamine:				
1.	2/1 (20 μg PA)	1/0 (40 μg PA)	2.5 (PA)	12/10

PA = injected into pulmonary artery

RES = placed in reservoir

CHAPTER I. SUPPLEMENTARY DISCUSSION

The Perfusate

The perfusate used in the isolated lung experiments reported above was designed to reproduce, as closely as possible, the concentrations of electrolytes, glucose, and colloids found in rat plasma (1). Many types of artificial perfusates have been used to perfuse isolated organs of animals, including organs taken from rats (2). However, none of the perfusates previously used has been specifically designed for the maintenance of optimal reactivity of the pulmonary vessels of the rat.

Although the concentrations of the constituents in the present perfusate are quite close to those of normal rat plasma, a few differences are apparent. Magnesium, normally present in rat plasma at a concentration of 1.3 mEq/L, has been omitted from the perfusate in my experiments. Calcium, normally present in rat plasma at a concentration of 6.2 mEq/L, has been reduced to 5 mEq/L in the perfusate. It has been reported that calcium and magnesium sometimes act synergistically in the maintenance of smooth muscle reactivity in perfused isolated organs (2). For this reason, the amounts of both magnesium and calcium were varied, in a series of experiments, to establish optimal concentrations in the perfusate for maximal vascular reactivity. Maximal vascular reactivity to pressor agents and minimal tendency to develop pulmonary edema occurred when the sum of the concentrations of magnesium and calcium was equal to 5 mEq/L. At higher concentrations of these electrolytes (as high as 17 mEq/L total) the tendency to develop pulmonary edema was very low, the lungs could be perfused for many hours, but the pulmonary vascular reactivity was reduced. At low concentrations of these electrolytes (as low as 3 mEq/L

total) the vascular reactivity was very high, but rapid formation of pulmonary edema ended the experiment within a few minutes. Magnesium and calcium were additive in these effects, and no other effect of magnesium was observed. Subsequent experiments utilized perfusates containing calcium (5 mEq/L) but no magnesium.

Colloid is an important constituent in the perfusate, without which one can expect the rapid development of edema. Dextran has been used with some success to provide colloidal osmotic pressure in artificial perfusates (3); however, edema forms relatively rapidly in comparison to perfusates employing albumin. In the present study, dextran (75,000 molecular weight) was used in two experiments at a concentration of 5 g/100 ml. In these experiments, the addition of angiotensin II to the perfusate shortly after beginning perfusion resulted in subsequent hypoxic pulmonary vasoconstriction. However, after the first 30 minutes of perfusion edema formation terminated each experiment.

Thus, while albumin is not necessary for the effect of angiotensin upon the hypoxic response, it is effective in avoiding edema formation during perfusion, and was used in subsequent experiments at a concentration of 5 g/100 ml.

Histamine

One of the initial objectives of this study was a more direct assessment of the role that histamine may play in hypoxic pulmonary vasoconstriction. Three different approaches were used, and although the studies were incomplete and did not absolutely rule out histamine as a mediator of the hypoxic response, they did suggest that pursuing the question of the role of histamine would be less productive than defining the role of other

possible mediators.

The first approach was to observe the effects upon the hypoxic response of adding histamine to the perfusate. The data from some of these experiments are reported in table 4 of the previous section. The addition of histamine had no effect on the hypoxic response, and amounts ranging from 10 to 100 μg (injected into the pulmonary artery) produced vasodilation and a fall in pulmonary artery baseline systolic pressure of 2 to 3 mm Hg. In other experiments of the same series (not reported in table 4), the injection of histamine into the pulmonary artery (in amounts ranging from 10 to 100 μg) during a peak vasoconstrictor response to angiotensin or hypoxia resulted in vasodilation and a partial reversal of the vasoconstrictor response. The magnitude of the decrease in pulmonary artery pressure was dependent upon the amount of histamine injected, and varied from 1 to 8 mm Hg mean pressure drop (6 experiments with 21 observations). During a hypoxic response, as the dilator effect of added histamine subsided, the pulmonary artery pressure returned to its pre-histamine-addition value and was terminated by return to normoxia in the usual manner. An example of this effect of histamine during a vasoconstrictor response to hypoxia is seen in figure 1.3. Barer, (4), in 1966, showed that small amounts of histamine (.4 to 1.8 ug) injected into the pulmonary artery of collapsed cat lungs produced vasodilation, where as larger amounts (.7 to 18 μg) produced vasoconstriction. Hauge (5), as mentioned above, could not demonstrate histamine-produced-vasoconstriction in rat lungs unless large amounts (up to 8 mg) were injected into the pulmonary artery; these large amounts of histamine also produced pulmonary edema. He did not report a dilator effect of smaller doses of injected histamine.

The effect of antihistamines in blocking the hypoxic response provides the strongest evidence for the postulated role of histamine as the

mediator. Therefore I investigated the effect of antihistamines upon the hypoxic response and on the vasodilation caused by histamine. Diphenhydramine was used in three experiments. In the first two of these, 4 mg/80 ml of diphenhydramine reduced the hypoxic response by an average of 66%. In the third, 2 mg/80 ml of diphenhydramine reduced the hypoxic response by 75%. The vasodilation caused by histamine was only slightly reduced. In one experiment, 5 mg/80 ml of purified chlorpheniramine reduced the hypoxic response by 25%, and completely blocked the dilator effects of injected histamine. In view of the dilator activity of histamine, the ability of the antihistamines to block the hypoxic response might be related to an action that is separate from their ability to block histamine. Because diphenhydramine, which has more central nervous system and atropine-like side effects than chlorpheniramine but only 1/20 the antihistaminic activity (6), is most effective in blocking the hypoxic response, atropine, which has been previously reported to have no effect upon the hypoxic response (7), was tested in three experiments. In the first two of these, 4 mg/80 ml of atropine reduced the hypoxic response by an average of 57%. In the third, 5 mg/80 ml of atropine reduced the hypoxic response by 59%. The vasodilator effect of histamine was not affected by atropine. These observations, although in need of verification in a larger series of experiments, suggest that the mechanism of action of antihistamines in blocking the hypoxic response may be nonspecific and would be difficult to assess.

The last series of experiments that relates to the effect of histamine during hypoxia was designed to estimate the amount of histamine which may be released during hypoxia. It was thought that if enough histamine is released during hypoxia to effect vasoconstriction its con-

centration in the perfusate might reach detectable levels. The principles of the assay that was used to estimate histamine concentration in the artificial perfusate was described by Miller in 1969 (8). This assay uses an enzyme, histamine-methyl-transferase extracted from guinea pig brains (9), to attach a ^{14}C labeled methyl group to the histamine in the perfusate. A small quantity of ^{3}H labeled histamine, added to the perfusate as a tracer, is also methylated with the labeled methyl group. After the methylating reaction is complete, the labeled histamine is extracted into chloroform, the chloroform is evaporated, and the labeled histamine is taken up into a toluene phosphor. The samples are then counted in a liquid scintillation counter. The amount of histamine in the unknown samples is directly proportional to the ratio of $^{14}C/^{3}H$, and can be calculated from a standard curve prepared simultaneously with the samples. The assay is specific for histamine, and can detect concentrations as low as 2 ng/ml of sample. Figure 1.4 presents an outline of the assay. In four experiments, perfusate samples were collected (A) before the addition of angiotensin II (when hypoxic responses were not present), (B) during the peak of a prolonged (5 minutes) hypoxic response, (C) during the return of the pulmonary artery pressure to baseline after a hypoxic response was terminated, and (D) at the end of the experiment after histamine was added to the perfusate, at a concentration of 40 ng/ml, as an internal standard. These samples were collected from the left atrial cannula, and were assayed for histamine content. Histamine was not detected in any sample except the internal standard added to sample number four. In these experiments, histamine either was present in concentrations too low to be detected by the assay method used, or was not released during hypoxia.

Figure 13: Effect of histamin injected into the pulmonary artery at the peak of a hypoxic response in the isolated rat lung.

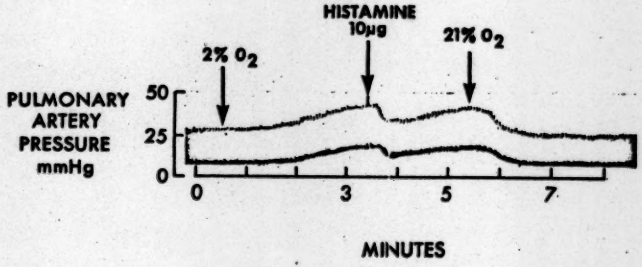

Figure 1.4

perfusate sample (.4 ml)

add: S-adenosyl-l-methionine-methyl-^{14}C (40 nanocuries - 220 ng),
histamine-^{3}H (10 nanocuries - 0.3 ng),
histamine-methyl-transferase (2 mg), and
.05 M. $NaPO_4$ buffer (pH 7.9) to total volume of 1 ml.
incubate at 37°C for 1 hour.

add: 1 N. NaCl saturated NaOH (2 ml), and
saturate with NaCl.

extract with 8 ml chloroform.
centrifuge at 250 X G for 5 minutes.

aspirate and discard the water phase.
to the organic phase add 1 N. NaCl saturated NaOH (2 ml).
centrifuge at 250 X G for 5 minutes.

aspirate and discard the water phase.
aspirate and evaporate the organic phase.
dissolve the residue from the organic phase in 95% ethanol (1 ml), and
toluene phosphor (10 ml).

count samples in liquid scintillation counter.

Histamine may play a role in the vasoconstrictor response to hypoxia as suggested by Hauge. Another approach to the definition of such a role would be to study histamine turn-over during hypoxia in pulmonary tissue since it may be the rate of formation rather than the static concentration which is important. This type of study could be done in the isolated rat lung utilizing the artificial perfusate and isotope labeled histidine.

A specific agent required for hypoxic pulmonary vasoconstriction has not been previously demonstrated in vitro or in vivo. The present study has demonstrated a primary role for angiotensin II in the mechanism underlying the hypoxic response in isolated non-blood perfused rat lungs. However, a specific requirement that angiotensin II be present for the hypoxic response to occur in vivo, as discussed above, was purely speculative. In order to demonstrate such a requirement, one must first develop a reliable in vivo preparation in which reproducible hypoxic pulmonary vasoconstriction can be obtained; and second, a method must be available to demonstrate a specific necessity for angiotensin II in the hypoxic response.

The question of whether angiotensin II is utilized in vivo to effect hypoxic responses was investigated as described in the following chapter.

REFERENCES

1. Dittmer, D.S. (Ed.), Blood and Other Body Fluids. Federation of American Societies for Experimental Biology, Washington, D.C. 1961.

2. Norman, J.C. (Ed.), Organ Perfusion and Preservation. Appleton-Century-Crofts, N.Y., 1968.

3. Daly, I. de B., and Hebb, C., Techniques for the artificial perfusion of the lungs. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., 350, 1966.

4. Barer, G.R., Reactivity of the vessels of collapsed and ventilated lungs to drugs and hypoxia. Circulation Res. 18. 366-378. 1966.

5. Hauge, A., Role of histamine in hypoxic pulmonary hypertension in the rat: blockade or potentiation of endogenous amines, kinins, and ATP. Circulation Res. 22. 371. 1968.

6. Goodman, L.S., and Gilman, A., Pharmacologic Basis of Therapeutics. 3d. ed. MacMillan Co., N.Y. 636-637. 1965.

7. Logaras, G., Further studies of the pulmonary arterial blood pressure. Acta Physiol. Scand. 14. 119-135. 1947.

8. Miller, R.L., McCord, C., Sanda, M., Bourne, H.R., and Melmon, K.L., Application of the enzymatic double isotope dilution assay for the study of histamine in plasma. J. Pharmac. Exp. Therap. 175. 228. 1970.

9. Brown, D.D., Tomchick, R., and Axelrod, J., The distribution and properties of a histamine methylating enzyme. J.B.C. 234. 2948. 1959.

CHAPTER 2. USE OF P-113

Angiotensin II is an octapeptide; the first position in the chain is occupied by an aspartyl residue, and the last position by phenylalanine. Steric alteration of the terminal phenylalanine results in loss of biologic activity; steric alteration of the aspartyl residue results in a prolongation in duration of action, presumably due to decreased enzymatic degradation (1). Recently, a number of analogues of angiotensin II, substituted in the one and eight positions, have been tested for their ability to block the actions of angiotensin II (2,3). One of these analogues, 1-sarcosine-8-alanine-angiotensin II (also known as P-113), has been found to be an effective and specific competitive inhibitor of the pressor and adrenal steroid-secreting effects of angiotensin II (4).

Isolated Lung

In five experiments P-113 was tested for its affect upon hypoxic responses in isolated perfused rat lungs. These experiments utilized the preparation and procedures described in the previous chapter. Angiotensin II and P-113 were added to the perfusate reservoir in varying concentrations at different times throughout each experiment.

Results

In one experiment, the initial administration of P-113 (20 µg/80 ml) blocked both the vasoconstrictor effect of 1 µg of angiotensin II without hypoxia and the subsequent development of hypoxic responses. Ten µg of angiotensin II administered 40 minutes later without hypoxia produced a small degree of vasoconstriction, and small responses to hypoxia. In four other experiments, the initial administration of 1 µg of angiotensin

II resulted in subsequent hypoxic responses. The disappearance of these hypoxic responses with time (described in the previous chapter) was not hastened by the subsequent administration of 10 μg of P-113. In three of these experiments, angiotensin II (in 1 to 10 μg amounts) was again administered, but did not restore the hypoxic responses until enough time had elapsed for partial reversal of the inhibition caused by P-113 (30 to 60 minutes).

The results of one such experiment are illustrated in Figure 2.1. The addition of 1 μg of angiotensin II produced vasoconstriction, and hypoxic responses that decayed over the course of 90 minutes. The addition of 10 μg of P-113 after 50 minutes did not hasten the decay of the response, but did completely block the vasoconstriction and augmentation of hypoxic responses by a subsequent addition of 1 μg of angiotensin II. 75 minutes after the addition of P-113, 10 μg of angiotensin II caused a small degree of vasoconstriction and a small augmentation of hypoxic responses.

In Vivo

The successful use of P-113 in blocking the hypoxic response *in vitro*, and the characterization of its effects, provides a background for its use *in vivo*. However, a number of considerations concerning the *in vivo* preparation, as well as the use of P-113 in such a preparation must be made.

Preparation

As discussed in Chapter 1, five variables must be measured or controlled in any preparation in order to demonstrate primary pulmonary vasomotor responses. These variables are: cardiac output; systemic back pressure changes; changes in bronchial circulation; effects of changes

in ventilation on pulmonary vessels; and bronchomotor effects. An anesthetized rat with pulmonary artery pressure, left atrial pressure, and thoracic aorta blood flow measured, and which is ventilated with the positive pressure arrangement described in chapter 1, would have all the variables measured or controlled except changes in bronchial circulation. As pointed out by Daly and Hebb, the ratio of bronchial blood flow to pulmonary blood flow is generally much smaller in the intact animal than in those perfused preparations which have an intact bronchial circulation. They therefore concluded that it is probably not an important source of error in the estimation of pulmonary vascular resistance in intact animals (5). Furthermore, Thilanius concluded that changes in bronchial circulation could not have been responsible for the pulmonary vascular changes observed in intact dogs during acute hypoxia (6). Because of these observations, and because the parameters to be measured, pulmonary artery pressure and thoracic aorta blood flow, would reflect overall resistance to blood flow through the lung, I felt that a small contribution from the bronchial circulation, if present, would not be an important source of error.

A potential source of difficulty in estimating cardiac output would come from alterations in sympathetic and parasympathetic activity to the heart and peripheral vessels during hypoxemia. Such feed-back effects would cause rapid alterations in cardiac output and obscure the direct effects of hypoxia on pulmonary vascular resistance. Changes in baroreceptor, chemoreceptor, and central nervous system influences have been successfully minimized by the use of ganglionic blocking agents in intact rat preparations (used as bioassay systems for vasoactive agents) (7). Pentolinium tartrate is the ganglionic blocking agent most frequently

used in these preparations, and was chosen for use in the present study.

Thoracic aorta blood flow was measured using a linear output pulsatile Biotronics 610/flow meter with a non-cannulating blood flow transducer (2 mm internal diameter). Because of the inherent difficulties in calibrating the flow meter, and because the important variable to be measured was changes in cardiac output during hypoxia rather than the actual value of the cardiac output, changes were estimated by comparing heart rate and stroke volume before and during a hypoxic stimulus. Heart rate was counted over a ten second control period just prior to the initiation of hypoxia, and the area under each stroke volume curve was integrated over this same period. Similar measurements were made during the peak of a hypoxic response, and the product of heart rate times stroke volume for each of these periods was compared. In this manner, any changes in blood pressure across the pulmonary circulation (pulmonary artery pressure minus left atrial pressure) caused by changes in cardiac output during hypoxia could be calculated, and an estimate of changes in pulmonary vascular resistance obtained. Thus, if the blood pressure across the pulmonary circulation increased from ten mm Hg to 20 mm Hg (by 100%) during hypoxia but the cardiac output increased by ten percent, the increase in blood pressure resulting from vasoconstriction was actually nine mm Hg.

In <u>in vivo</u> preparation was a Sprqgue Dawley rat of either sex (300 g) that was anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg). A tracheostomy was performed and the chest opened during positive pressure ventilation as described in Chapter 1. The pulmonary artery pressure was measured from a 22 gage needle placed directly into the pulmonary artery. The left atrial pressure was measured from a plastic cannula (PE# 90) placed directly into the left atrial appendage and secured

with a suture. The flow transducer was passed into the chest through an incision in the left lateral rib cage, and placed around the thoracic aorta. The pulsatile aortic blood flow, and the pulmonary artery and left atrial pressures (using Statham p23AA pressure transducers) were recorded on a Grass model 5d recorder. Drugs diluted in saline were administered through a plastic cannula (PE# 90) placed in the right external jugular vein. At the beginning of each experiment, 200 U.S.P. units of heparin and pentolinium tartrate (Wyeth) 8 mg/kg were administered through the venous cannula. The lungs were ventilated 10 ml/cycle of room air at a frequency of 60 cycles/minute. To produce alveolar hypoxia the lungs were ventilated for 1 1/2 minutes with 5% O_2 and 95% N_2.

Results

The initial experiments *in vivo* were designed to characterize the hypoxic response. Hypoxic tests were performed every 10 minutes throughout the experiment. The results of these experiments are reported in table 2.1, and show that the hypoxic response *in vivo* increases in magnitude with each hypoxic test to reach a maximum after about 40 minutes. Thereafter, the response stays at the plateau level throughout the remainder of the experiment. If exposure to hypoxia is stopped for 1 hour or longer, the subsequent hypoxic response will be greatly reduced from the plateau value, but will again build up to plateau with repeated hypoxic tests. One explanation for these results is that the lung *in vivo* has the ability to utilize increasing amounts of angiotensin (perhaps over the same concentration range as delineated *in vitro*) when repeatedly challenged by hypoxic stimuli; the disappearance of the hypoxic response, after an hour free of exposure to hypoxia, may be caused by disassociation

of angiotensin-receptor complexes previously formed.

In five subsequent experiments, P-113 was administered to intact animals at a different concentration for each animal. The drug was administered at each biologic half-life (approximately 12 minutes (8)) until maximal reduction of the hypoxic responses was achieved. The results, reported in table 2.2, show that P-113 effectively reduced the hypoxic responses. The time to maximal reduction of the hypoxic responses was an average of 40 minutes with a range of 30 to 50 minutes. After stopping the administration of P-113 the hypoxic responses were seen to return toward their plateau values after 3 to 5 half-lives (36 to 60 minutes).

Figure 2.2 illustrates similar results in a single animal. The initial hypoxic response was 4 mm Hg, but increased over 40 minutes (5 hypoxic tests) to a plateau height of 7 mm Hg. The addition of P-113 in 100 μg quantities at each half-life over a 40 minute period resulted in an 86% reduction of the plateau responses. One hour after stopping the administration of P-113, the hypoxic responses had returned to 5.7 mm Hg (81% of the plateau value).

Since plateau hypoxic responses are reached after 40 to 50 minutes (4 to 5 hypoxic tests), a rough estimate of the increment of angiotensin II accumulated in the lung following each hypoxic test would be 2 to 3 μg. These numbers are derived by assuming that the same maximally effective concentration as previously delineated for the isolated lung (10 to 12 μg total) is also effective *in vivo*. If the plateau responses are maintained by these periodic increments of angiotensin II, one would predict that 10 μg of P-113 administered every half-life would reduce the plateau responses by approximately 50% assuming that 20% of the total plasma volume (9), and thus 20% of the administered amount of P-113, is

contained in the lung at any one time. Similarly, 25 μg of P-113 would reduce the responses by 70%, and 90 μg by 90%. These predictions assume that the receptor affinity is essentially equal for both angiotensin II and P-113. The observed values reported in table 2.2 are quite close to the predicted values.

Thus, hypoxic pulmonary vasoconstriction in intact rats can be blocked by the administration of P-113.

Summary

The *in vitro* and *in vivo* findings can be summarized as follows:

1. Angiotensin II is required for hypoxic pulmonary vasoconstriction in isolated perfused rat lungs.
2. This effect is separate from its direct vasoconstrictor action and operates over an approximate perfusate concentration range of 12 to 120 nM.
3. Other vasoactive agents tested do not produce or affect the hypoxic response, and their vasoactivity does not depend upon angiotensin II.
4. Development of hypoxic responses in isolated lungs is blocked by P-113, a competitive inhibitor of angiotensin II.
5. Hypoxic responses in intact rats are reduced by the systemic administration of P-113 in appropriate concentrations.

Discussion

These findings, and the time course of development, blockade, and disappearance of the hypoxic response, suggest that angiotensin II is utilized during a hypoxic stimulus to effect pulmonary vasoconstriction in the rat. The mechanism of this action of angiotensin II is not clear from these studies, but the results are consistent with the hypothesis that it is an action of angiotensin II that is separate from its direct

vasoconstrictor effect, and depends upon continuous occupancy of receptors. It may be that angiotensin II binds to the receptors and augments the hypoxic response until the angiotensin-receptor complex is dissociated. If so, the dissociation takes an observed average of 30 to 60 minutes. After this time, the inhibitor may associate with the receptors and block further augmentation of hypoxic responses by angiotensin II until the inhibitor-receptor complex is dissociated (also an observed average of 30 to 60 minutes).

An alternative hypothesis to explain the observed effects of P-113 on hypoxic responses is that a specific angiotensin II receptor exists in the lung that is responsible for producing hypoxic pulmonary vasoconstriction. This receptor may have a low accessibility or affinity for P-113, thus requiring the presence of large amounts of P-113 for extended periods of time to achieve blockade and reduction of hypoxic responses.

Where these postulated receptors are located, and how the angiotensin-receptor complex allows hypoxic pulmonary vasoconstriction is not known. Ryan has demonstrated that angiotensin I converting enzyme activity is located in the pulmonary vascular endothelial cells, and that angiotensin II is rapidly formed without being retained by the lungs (10). It may be that angiotensin II is formed and bound at the proper site only during hypoxic stimulation. Once bound, the angiotensin-receptor complex may produce a conformational change in the vascular smooth muscle membrane which allows electrolyte shifts, such as those postulated by Bergofsky and Holtzman (discussed in the introduction section), of sufficient magnitude to effect vasoconstriction during hypoxia. Alternatively, angiotensin II may be bound in the perivascular lung tissue to allow the release or action of another vasoactive agent.

These hypothesis have not been tested in the present series of experiments, however, mutually exclusive hypotheses formulated around these questions might be differentiated by a number of different experimental approaches. For instance, the use of isolated pulmonary artery smooth muscle strips in a hypoxic organ bath containing angiotensin II would show whether this combination was sufficient for hypoxic pulmonary vasoconstriction, or whether another agent was required. The use of isotope labeled angiotensin II during hypoxia in isolated lungs, with subsequent cell fractionation might provide evidence for where and under what conditions angiotensin is bound. Another approach to the questions surrounding the ability of the lung to form and utilize angiotensin II in vivo to effect the hypoxic response would be to use both nephrectomized and unilateral renovascular hypertensive rats in the in vivo preparation previously described. Comparison of the hypoxic reactivity of the lungs from these groups of animals could provide useful information with regard to whether renin from the kidney, or angiotensin II from the blood play an important part in hypoxic pulmonary vasoconstriction.

Studies of the type outlined above, with corollary clinical investigations, may lead to a better understanding of the pathogenesis and therapy of hypoxic pulmonary hypertension in man.

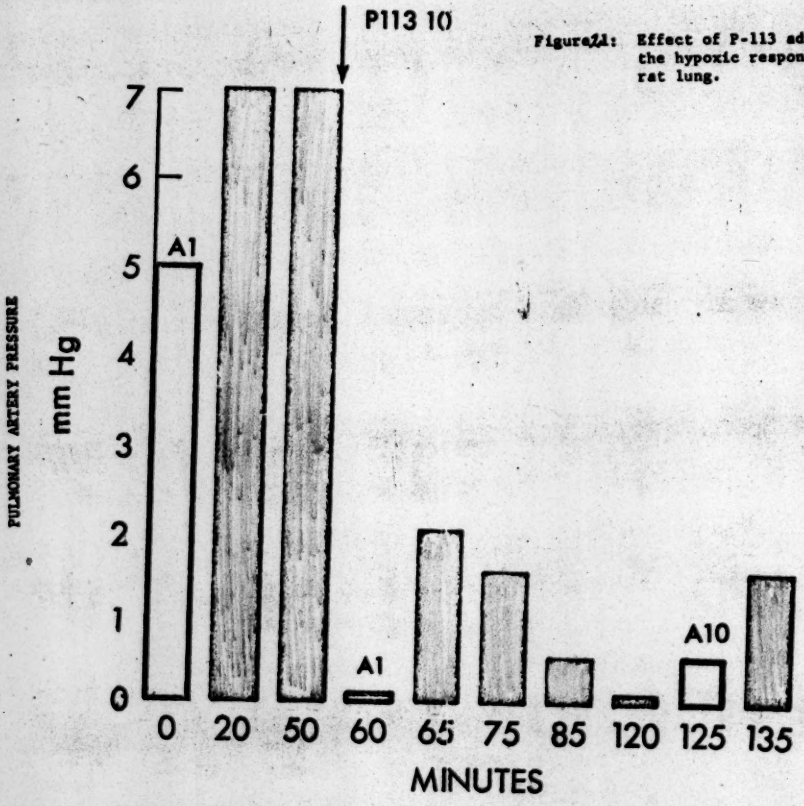

Figure 1: Effect of P-113 administration upon the hypoxic response in the isolated rat lung.

Figure 20: Effect of P-113 administration upon the hypoxic response in the intact rat.

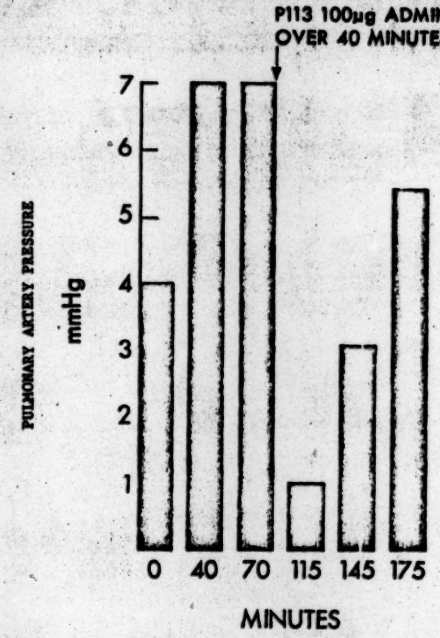

Table 1: Characterization of hypoxic responses in intact rats. Hypoxic tests performed every 10 minutes.

EXPERIMENT	INITIAL RESPONSE mm Hg	PLATEAU RESPONSE mm Hg	TIME TO PLATEAU MINUTES	DURATION OF PLATEAU MINUTES
1.	1.8	6.6	48	remainder of experiment (85 MIN.)
2.	1.0	4.5	60	remainder of experiment (30 min.)
3.	0.9	5.0	30	remainder of experiment (90 min.)
4.	2.0	5.0	35	remainder of experiment (70 min.)

Table 2. Reduction of hypoxic responses in vivo by P-113 administered every half-life.

Exp.	Plateau Resp. mm Hg	Amt. of P-113 ug	Reduced resp. mm Hg	% Reduced	Time to Max. Reduction minutes
1.	8	10	3.5	56	45
2.	12	15	4.0	66	40
3.	9	30	2.7	70	30
4.	12	40	3.0	75	30
5.	7	100	1.0	86	50

REFERENCES

1. Wilson, C.O., Gisvold, O., and Doerge, R.F., Textbook of Organic Medicinal and Pharmaceutical Chemistry. Fifth Edition, J.B. Lippincott Company, Philadelphia and Toronto, p. 842, 1966.

2. Pals, O.T., Masucci, R.D., Sipos, F., and Denning, G.S., A specific competitive antagonist of the vascular action of angiotensin II. Circulation Res. 29. 664. 1971.

3. Türker, R.K., Hall, M.M., Yamamoto, M., Sweet, C.S., Bumpus, F.M., A new, long-lasting competitive inhibitor of angiotensin II. Science. 29 Sept. 1972. 177 Number 4055, 1203.

4. Johnson, J.A., and Davis, J.O., Effects of an angiotensin II analog on blood pressure and adrenal steroid secretion in dogs with thoracic caval constriction. The Physiologist. 15 Number 3. 184. 1972.

5. Daly, I. de B., and Hebb, C., Criteria for the demonstration of primary pulmonary vasomotor responses. In Pulmonary and Bronchial Vascular Systems. Baltimore, Williams and Wilkins Co., pp. 172-173. 1966.

6. Thilenius, O.G., Effect of anesthesia on response of pulmonary circulation of dogs to acute hypoxia. J. Appl. Physiol. 21. 901-904. 1966.

7. Lee, M.R., Cook, W.F., McKenzie, J.K., A sensitive method for assay of plasma renin activity. Circulation Res. 19. 260-268. 1966.

8. Pals, D.T., Mosucci, F.D., Denning, G.S., Sipos, F., and Fessler, D.C., Role of the pressor action of angiotensin II in experimental hypertension. Circulation Res. 29. 673-681. 1971.

9. Ganong, W.R., Medical Physiology. 3rd Edition, Lang Medical Publications, Los Altos, California, p. 523. 1967.

10. Ryan, J.W., Smith, U., and Niemeyer, R.S., Angiotensin I: metabolism by plasma membrane of lung. Science. 176. 64. 7 April 1972.